Melanopsin Mediated Post-Illumination Pupil Response in Early Age-related Macular Degeneration

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ABSTRACT

Purpose: To determine whether melanopsin expressing intrinsically photosensitive Retinal Ganglion Cell (ipRGC) inputs to the pupil light reflex (PLR) are affected in early age-related macular degeneration (AMD).

Methods: The PLR was measured in 40 participants (20 early AMD and 20 age-matched controls) using a custom-built Maxwellian-view pupillometer. Sinusoidal stimuli (0.5 Hz, 11.9 s duration, 35.6° diameter) were presented to the study eye and the consensual pupil response was measured for stimuli with high melanopsin excitation (464nm; blue) and with low melanopsin excitation (638 nm; red) that biased activation to the outer retina. Two melanopsin PLR metrics were quantified: the Phase Amplitude Percentage (PAP) during the sinusoidal stimulus presentation and the Post-Illumination Pupil Response (PIPR). The PLR during stimulus presentation was analyzed using latency to constriction, transient pupil response and maximum pupil constriction metrics. Diagnostic accuracy was evaluated using receiver operating characteristic (ROC) curves.

Results: The blue PIPR was significantly less sustained in the early AMD group ($p<0.001$). The red PIPR was not significantly different between groups ($p>0.05$). The PAP and blue stimulus constriction amplitude were significantly lower in the early AMD group ($p < 0.05$). There was no significant difference between groups in the latency or transient amplitude for both stimuli ($p>0.05$). ROC analysis showed excellent diagnostic accuracy for the blue PIPR metrics (AUC>0.9).

Conclusions: This is the initial report that the melanopsin controlled PIPR is dysfunctional in early AMD. The non-invasive, objective measurement of the ipRGC controlled PIPR has excellent diagnostic accuracy for early AMD.

Keywords: intrinsically photosensitive Retinal Ganglion Cells (ipRGCs), melanopsin, pupil light reflex, post-illumination pupil response

INTRODUCTION

Melanopsin-expressing intrinsically photosensitive Retinal Ganglion Cells (ipRGCs) form the recently identified third photoreceptor class in the eye and have important non-image forming functions including mediation of the pupillary response[1, 2] and photoentrainment of circadian rhythm.[3-6] Their cell bodies are primarily located in the ganglion cell layer, with a small number displaced to the inner nuclear layer.[7] While ipRGC physiology and function has been studied in both nocturnal rodents[5, 8] and primates,[9, 10] research is increasingly focusing on their roles in diurnal humans and in particular in diseased eyes, with preliminary reports for potential ipRGC dysfunction in age-related macular degeneration (AMD).[11]

Psychophysical and electrophysiological studies are well established for measuring functional deficits of rods and cones in different stages of AMD[12-19] however, the effect of AMD on ipRGC function is unknown. The Pupil Light Reflex (PLR) provides a rapid, objective, non-invasive measure of both inner (ipRGC) and outer (rod and cone) retinal function.[6, 11, 20-25] Following onset of an incremental light pulse, the initial PLR is mediated by the outer retina[20, 26] with increasing melanopsin input with longer stimulus durations,[20] while ipRGCs control the Post-Illumination Pupil Response (PIPR), the sustained pupil constriction after light offset.[2, 11, 25] Recently it was demonstrated that for sinusoidal lights with high melanopsin excitation, the peak-to-trough amplitude of the phasic PLR during flicker stimulation was suppressed compared to lights with low melanopsin excitation.[11, 27] This suppression is analysed using a Phase Amplitude Percentage (PAP) metric to provide a direct marker of melanopsin inputs to the pupil during light stimulation.[11]

IpRGC function has been measured using the PIPR in diabetic patients without diabetic retinopathy,[28] glaucoma,[29-31] retinitis pigmentosa,[6, 32, 33] Leber's hereditary optic neuropathy[34] and retinal dystrophy.[35] In AMD, the PLR has been used as a measure of outer retinal function[11, 36-41] and demonstrates a longer latency to constriction and reduction in maximum

pupil constriction amplitude. However, these studies were not designed to measure ipRGC function in AMD. Pathological changes in AMD first occur in the paracentral retina[42] where ipRGCs have their highest density,[9, 10] which may make ipRGCs susceptible during early disease. In advanced stages of the disease, there is ~50% loss of inner retinal ganglion cells.[43] Although ipRGCs are robust in early stages of diseases affecting the optic nerve,[44] it is still not known how ipRGCs are affected in patients with early AMD, with our group showing the first evidence of ipRGC alteration.[11] The primary purpose of this study is to measure the effect of early AMD on inner retinal contributions to the PLR using the PIPR metric and to use a novel sinusoidal stimulus paradigm that reflects inner retina (ipRGCs) and outer retina (rods and cones) interactions in the phasic pupil response.[11]

METHODS

Participants

Forty participants (20 female, 20 male) were recruited from the Queensland University of Technology (QUT) eye clinic. Twenty participants (10F and 10M; age 69.3 ± 5.5 years) were healthy controls and 20 were participants with early AMD (10F and 10M; age 72.9 ± 6.3 years) who had either AREDS grade 2 or grade 3 AMD (Table 1) based on the results of two independent gradings of the fundus photographs.[45] Where early AMD was present in both eyes, the patients preferred eye was measured. Where participants had grade 1 in one eye, the eye with early AMD was chosen as the study eye. Participants with Grade 4 (advanced) in either eye were excluded. All participants underwent an ophthalmic examination, which included visual acuity (Bailey Lovie), ophthalmoscopy, colour vision (Lanthony D-15 desaturated), tonometry (iCare TA01, Finland), optical coherence topography (OCT, Cirrus HD-OCT; Carl Zeiss Meditec, USA) and colour fundus photography (CR-1, Canon, Australia). The control group had normal vision (6/6 or better), crystalline lens opacities ≤ Grade 2,[46] no ocular disease and were in good general health. The early AMD group had a

best corrected visual acuity ≥ 6/9 in the study eye, crystalline lens opacities ≤ Grade 2[46] and no history of ocular or systemic disease other than AMD. No participant had taken any medication that could affect the pupil response. Written informed consent was obtained from all participants and the study was conducted in accordance with the requirements of the Queensland University of Technology Human Research Ethics Committee and the tenets of the Declaration of Helsinki.

Table 1: Grading of each eye for all AMD patients according to AREDS grading scale with the study eye in bold.

	Grade			Grade	
Px No.	RE	LE	Px No.	RE	LE
1	2b	**2b**	11	3a	**3c**
2	3d	**2a**	12	3a	**3a**
3	2c	**2b**	13	3c	**3c**
4	2b	**2b**	14	**2a**	2b
5	1	**2c**	15	**2b**	2b
6	3d	**2b**	16	**2c**	1
7	2b	**2b**	17	**2a**	3
8	2b	**2b**	18	**3c**	3c
9	2b	**2b**	19	**3a**	3d
10	3a	**3c**	20	**3c**	3c

Pupillometer

Sinusoidal stimuli (0.5 Hz, 11.9 s duration) were presented using a custom built, extended Maxwellian view pupillometer[11, 23] comprising narrowband LED light sources (638 nm and 464 nm) imaged in the pupil plane via two Fresnel lenses (100 mm diameter, 127 mm and 70 mm focal lengths; Edmund Optics, Singapore) and a 5˚ light shaping diffuser (Physical Optics Corp., California, USA) to provide a 35.6˚ diameter light stimulus (retinal image diameter: 15.4 mm).[25] The consensual pupil light reflex was recorded under infrared LED illumination (λ_{max} = 851 nm) using a Pixelink camera (IEEE-1394, PL-B741 Fire Wire; 640 x 480 pixels; 60 frames/s) through a telecentric lens (Computar 2/3″ 55 mm and 2 x extender C-Mount).[25] Customized Matlab software (version 7.12.0, Mathworks, Massachusetts, USA) controlled

stimulus presentation and timing. Blink artefacts were identified and extracted during software analysis of pupil recordings by a customized algorithm using linear interpolation.[25] The spectral outputs of the LED stimuli were measured with a Spectroradiometer (StellarNet, Florida, USA) and irradiance was calibrated with an ILT1700 Research Radiometer (International Light Technologies, Massachusetts, USA).

Procedure

After an initial ophthalmic assessment, Tropicamide 1% (Minims, Chauvin Pharmaceuticals Ltd., England) was instilled in the study eye and a 15 minute dark adaptation period commenced in a darkened (< 1 lux) laboratory prior to pupil recordings. The participant was then aligned in the pupillometer in Maxwellian view with the head held steady by temple bars and a head brace. The participant was instructed to look straight ahead in the dark as if fixating a distant object and fixation was monitored with the IR camera. The consensual pupil reflex was measured in response to short wavelength light (464nm) with high melanopsin excitation[2, 3, 21, 22] and to long wavelength light (638 nm) that biased activation to the outer retina[21] and provided a control. The corneal irradiance of the long and short wavelength stimuli was 15.1 log quanta.cm^{-2}.s^{-1}. This provided a retinal irradiance of 14.5 log quanta.cm^{-2}.s^{-1} for short wavelength and 14.9 log quanta.cm^{-2}.s^{-1} for long wavelength light.[47] A single pupil recording comprised a 10 s pre-stimulus period, presentation of an 11.9 s sinusoidal stimulus and a 40 s post-illumination period. Two repeats for each stimulus (464 nm and 638 nm) were recorded with a five minute dark adaptation period between trials.[27] The short and long wavelength stimuli were alternated in all sessions with the long wavelength light always presented first to control for the effect of melanopsin bistability.[48] All measurements were completed during a similar time of day to control for the effect of circadian variation on the PIPR.[49]

Analysis

Figure 1 shows the average pupil light reflex of 20 control participants with no retinal abnormalities in response to an 11.9 s, 0.5 Hz sinewave stimulus of long (638 nm; red) or short (464 nm; blue) wavelength light. The PLR was described with linear and exponential models[11, 49] and analyzed according to protocols defined by Adhikari, Zele and Feigl.[25] To control for individual differences in resting pupil diameter, all data are reported as a percentage of the resting baseline pupil diameter (average pupil diameter during 10 s pre-stimulus period). The PLR during stimulus presentation was quantified using the transient pupil response (maximum constriction at 500 ms after stimulus onset), latency to constriction (time taken to constrict 1% of baseline pupil diameter) and constriction amplitude (minimum pupil diameter during presentation of light stimulus); a smaller percentage value indicates larger constriction amplitude (Figure 1). The PIPR was quantified at 6 s (sustained pupil constriction at six seconds after light stimulus offset) and plateau (derived from the exponential model fit to the PIPR). The Phase Amplitude Percentage (PAP)[11] was calculated as the percentage difference in peak-to-trough amplitude between the phasic pupil response during light stimulation to the long and short wavelength sinewave stimuli.

Statistical analyses were performed using commercially available statistical software (IBM SPSS, version 21; IBM Corporation, Armonk, NY, USA). Parametric tests were applied to all data that passed the Kolmogorov-Smirnov test of normality. Each metric was evaluated by comparing red and blue stimulus responses within and between groups using repeated measures ANOVA and appropriate post-hoc analysis was performed when significant effects occurred. The latency to constriction was not normally distributed and an independent samples Mann-Whitney U test was used to compare between groups. The PAP was evaluated using independent samples t-test. A p-value of < 0.05 was considered statistically significant. The diagnostic accuracy of the PIPR metrics in determining early AMD was evaluated using receiver operator characteristic (ROC) analysis by quantifying the difference between the AMD patients and control participants.

RESULTS

Figure 2 shows the mean PLR and 95% confidence limits in response to long and short wavelength stimuli for the early AMD group compared to the healthy controls. Table 2 gives the PLR metrics for the early AMD group and control group. The AMD patients with AREDS grade 2 and grade 3 were not significantly different on any PLR metric and therefore the AMD data were pooled for comparison with the control group. The PLR to the blue stimulus was significantly different between groups for the 6 s PIPR ($F_{1, 39} = 64.56$, $p < 0.0001$; Figure 3A), plateau PIPR ($F_{1, 39} = 33.78$, $p < 0.0001$; Figure 3B) and maximum constriction ($F_{1, 39} = 8.69$, $p = 0.005$; Figure 4C) where the amplitude was significantly less for the early AMD group compared to the control group. There was no significant difference between groups in the transient pupil response ($F_{1, 39} = 0.89$, $p = 0.351$; Figure 4A) or latency to constriction ($p = 0.947$; Figure 4B) for the blue stimulus. The PLR to the red stimulus was not significantly different between groups for any metric ($p > 0.05$). There was a significant difference ($p < 0.05$) between the red and blue stimulus response for all metrics except for latency to constriction ($p > 0.05$). Analysis of the PAP (Figure 4D) showed a significantly lower average percentage in the early AMD group (29.5 ± 9.4 %) compared to the control group (38.4 ± 11.5 %; t (38) = 2.375, $p = 0.023$). The slope of the linear regression of the 6 s PIPR amplitude as a function of age was not significantly different from zero indicating that there was no effect of age on the PIPR ($R^2 = 0.113$, $F_{1,19} = 2.291$, $p = 0.147$). There was no significant relationship between visual acuity and the PIPR metrics. The ROC analysis showed that the blue stimulus had a larger AUC for both the 6 s PIPR (AUC = 0.963, $p < 0.001$) and plateau PIPR metric (AUC = 0.928, $p < 0.001$) compared to the red control stimulus (6 s: AUC = 0.660, $p = 0.083$; plateau: AUC = 0.401, $p = 0.298$) (Figure 5).

Table 2: Pupil light reflex (PLR) metrics (μ ± σ) in healthy controls and patients with early age-related macular degeneration

	Blue stimulus		Red stimulus	
	AMD	Control	AMD	Control
Latency to constriction (ms)	209.6 ± 88.4	211.4 ± 89.1	219.4 ± 83.1	217.5 ± 87.7
Transient pupil response (%)	20.7 ± 8.4	23.4 ± 9.4	18.4 ± 7.3	20.7 ± 7.3
Maximum constriction (%)	42.9 ± 5.3 *	38.7 ± 3.3	47.8 ± 5.1	45.1 ± 3.9
6 s PIPR (%)	80.1 ± 6.4 *	63.0 ± 7.3	90.0 ± 4.2	87.5 ± 4.1
Plateau PIPR (%)	92.0 ± 4.6 *	75.6 ± 11.7	96.5 ± 4.9	96.9 ± 3.3

*** $p < 0.05$**

DISCUSSION

This is the initial demonstration of a significantly reduced post-illumination pupil response in persons with early age related macular degeneration. These findings indicate that intrinsic ipRGC inputs to the pupil control pathway are altered in early AMD and the pupillometric measurement of the PIPR has excellent (AUC > 0.90) diagnostic accuracy for early AMD. By comparison, pupil parameters reflecting outer retinal contributions to the PLR (transient and latency)[10, 11, 26] were not significantly affected. However, the large stimuli used in this study were selected to optimize ipRGC activation[11] and would therefore be less sensitive to the presence of small, localized outer retinal deficits as can occur due to drusen.[50, 51]

The exact pathomechanisms in AMD are unclear, with known loss of conventional retinal ganglion cells (RGC) in advanced stages of AMD;[43] previous histological studies did not study ipRGCs as they have been only recently discovered.[7] The relative numbers of different subtypes of ipRGCs may vary between species. There are at least five ipRGC subtypes (M1 to M5) that have been identified in transgenic mouse models based on their dendritic stratification that varies across the outer and inner laminae of the inner plexiform layer (IPL).[9] IpRGC dendrites express melanopsin and have comparable photon capture to the soma [52]

while also receiving synapses from bipolar and amacrine cells for signaling between outer and inner retina.[9, 53, 54] There is evidence of at least three ipRGC subtypes in primates[10, 55] but it is unknown how these different subtypes are affected by retinal and optic nerve disease. In rodent studies of retinal disease, Royal College of Surgeons (RCS) dystrophic rats and P23H transgenic rats were used to investigate melanopsin cell function in retinitis pigmentosa (RP).[35, 56] One study showed that some ipRGCs were lost with disease progression while a significant number of ipRGCs survived into advanced stages of degeneration in the far peripheral retina.[35] A second study showed progressive loss in density, cell integrity and dendritic arborisation of ipRGCs in advanced stages of RP [56] consistent with initial findings of ipRGC dysfunction in advanced AMD.[11] A number of rodent[57-59] and human[60-62] studies show that ipRGCs are more resistant compared to conventional retinal ganglion cells in optic nerve disease and a recent study in a rat model showed that density and dendritic arborization does not change with age.[63] An example of this resistance to damage is shown in a study in patients with glaucoma that demonstrated the PIPR in patients with early glaucoma was similar to controls,[29] but lower in patients with advanced glaucoma.[29, 30] In patients with Leber's hereditary optic neuropathy (LHON), the sustained pupil response to blue light in the affected eye was similar to that in the healthy eye, suggesting a resistance to the intracellular metabolic disorder affecting the optic nerve caused by a genetic defect.[34, 64] This is confirmed in a histological study of LHON that showed relative sparing of ipRGCs compared to other retinal ganglion cells.[60] Whilst remaining robust to early changes in diseases affecting the optic nerve or peripheral retina,[44] we hypothesize that ipRGCs may be more vulnerable in diseases affecting the central retina such as AMD.[42] No histological study has investigated ipRGC distribution and potential loss in AMD and our research findings suggest that due to their restricted number and paracentral location,[10] ipRGC damage may become manifest early in the condition.

Previous studies of the pupil light reflex in AMD focused on the latency to pupil constriction, transient pupil response and maximum pupil constriction which is largely controlled by the

outer retina,[36-40] however these studies included patients with advanced exudative AMD and the deficit is expected to be larger in later disease stages. Using multifocal pupillography, Sabeti et al[65] found reduced pupil responses in patients with early AMD, however their pupil paradigm is not designed to measure ipRGC function. While outer retinal deficits may have been manifest in the patients with early AMD, our testing conditions were primarily aimed towards optimum ipRGC isolation. Smaller stimuli [66] with retinal irradiance below melanopsin threshold[6, 22, 39, 67] can be useful to also detect deficits in rod and cone function.

A number of metrics have been used to define ipRGC response, namely redilation velocity,[29, 49] 6 s PIPR,[21] plateau PIPR[2, 6] and area under curve (AUC).[24] In this study, we used the 6 s and plateau PIPR metrics to measure ipRGC controlled PIPR following a recent study by Adhikari et al[25] who demonstrated that these metrics show the lowest coefficient of variation for inter and intra-individual measurements. The newly defined PAP metric[11] that uses the phasic response during light stimulation may be also beneficial in measuring inner and outer retinal interactions. It is thought that the peak-to-trough amplitude for the short wavelength stimulus is lower than that of the long wavelength stimulus due to the contribution of ipRGCs to maintain pupil constriction when stimuli have high melanopsin excitation;[11, 26, 20] we hypothesise that if there is ipRGC loss or dysfunction in retinal disease, the capacity of ipRGCs to maintain pupil constriction during light stimulation will be reduced and result in a larger outer retinal phasic pupil response such that the phasic pupil response to the stimuli with high and low melanopsin excitation (e.g. blue and red lights) become more similar (i.e. a lower PAP). Hence, the lower PAP result in the early AMD group compared to the healthy controls may indicate the onset of altered inner and outer retinal interactions. It is known for psychophysical studies that rod-cone interactions measured under mesopic light levels may be an early marker of dysfunction in people with high risk genotype for AMD.[68] The differences in PIPR shown in this study are unlikely to be due to lens attenuation as participants were age-matched and those with lens grading above grade 2 were excluded, providing a true reflection of differences in inner retinal melanopsin function.

In conclusion, this is the initial demonstration of an alteration of ipRGC function as measured via the PIPR in early AMD. IpRGCs may be more vulnerable to disease affecting the central retina as opposed to those affecting the peripheral retina or optic nerve. Given that the PIPR is affected in early AMD and in glaucoma[11, 29] but not in for example Leber's hereditary optic neuropathy,[34] these differences in the ipRGCs mediated pupil responses may help to provide further insight into the disease pathomechanisms. The PAP findings may be a result of altered signaling between inner and outer retina.[11] Histological studies are required for better understanding of pathophysiological processes involving ipRGCs in AMD. This study demonstrates that pupillometry provides a rapid, non-invasive means of measuring the pupil response to quantify ipRGC function in early stages of AMD. The pupillometry paradigms introduced here have excellent diagnostic accuracy and may also be useful in monitoring disease progression.[11]

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FIGURE LEGENDS

Figure 1: Graphic representation of the pupil light reflex (PLR) to long and short wavelength stimuli in healthy controls. The average pupil light reflex of 20 control participants with no retinal abnormalities to an 11.9 second, 0.5 Hz sinewave stimulus of long (638 nm; red) or short (464 nm; blue) wavelength light. Data are presented as a percentage of the baseline pupil diameter (horizontal dashed line). The 6 s PIPR (vertical dashed line) measures the pupil diameter six seconds after light offset while the plateau PIPR (horizontal dotted line) shows the plateau of the exponential fit to the post-stimulus pupil diameter. The PAP is determined by the average peak to trough amplitude of the red and blue sinewave pupil response during stimulus presentation.

Figure 2: Average pupil light reflex (PLR) to long and short wavelength stimuli in early AMD patients compared to healthy controls. The average pupil light reflex to long (panel A; red) and short (panel B; blue) wavelength light of early AMD patients compared to healthy controls. Panel A shows the upper and lower 95% confidence limits for the healthy controls (shaded) and the mean response for the early AMD group (solid line). Panel B shows the confidence limits (shaded) and mean response (solid line) for both control and early AMD groups. The sustained response to blue light, measured at 6 s post-stimulus (vertical line), is significantly reduced in the early AMD group.

Figure 3: Post-illumination pupil response to red and blue stimuli in control and early AMD patients. The post-illumination pupil response to long wavelength (red) and short wavelength (blue) stimuli measured at 6 s after stimulus offset (panel A) and at the plateau of

the exponential fit to the pupil diameter (panel B). The amplitude is expressed as a percentage of the average pre-stimulus pupil diameter (baseline). The pupil response was compared between the control group (unfilled) and early AMD group (pattern filled). There was a significant difference in pupil response to the blue stimulus between groups for both 6 s and plateau PIPR metrics with no difference between groups for the red stimulus.

Figure 4: Average pupil light reflex metrics to red and blue stimuli for the control group and early AMD group. Each panel compares the PLR during presentation of red or blue stimuli for the control (unfilled) and early AMD (patterned fill) groups. The transient response (panel A) and maximum pupil constriction (panel C) are expressed as a percentage of the baseline pupil diameter while latency to constriction (panel B) is given in milliseconds (ms). The phase amplitude percentage (PAP) (panel D) is expressed as the percentage difference in peak-to-trough amplitude between the phasic response to long and short wavelength sinewave stimuli. Both maximum constriction to the blue stimulus and PAP are significantly reduced in the AMD group compared to the control group. There is no significant difference in transient response or latency to constriction between groups for red and blue stimuli.

Figure 5: Receiver Operating Characteristic (ROC) curves for blue and red stimulus conditions for the 6s and plateau PIPR metrics. The sensitivity (true positive rate) is plotted as a function of specificity (false positive rate) for 6 s (panel A) and plateau (panel B) PIPR measurements. The blue stimulus (blue line) shows a significantly higher sensitivity and specificity with a larger AUC compared to the red stimulus (red line) for both metrics.

Figure 1

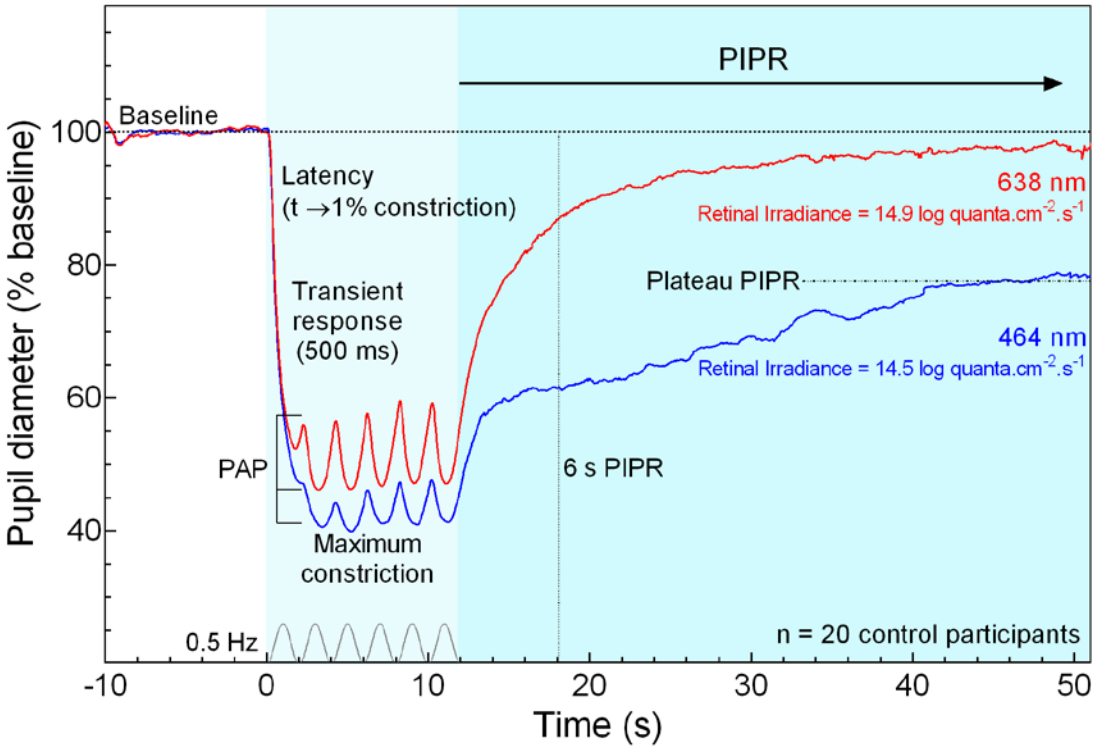


Figure 2

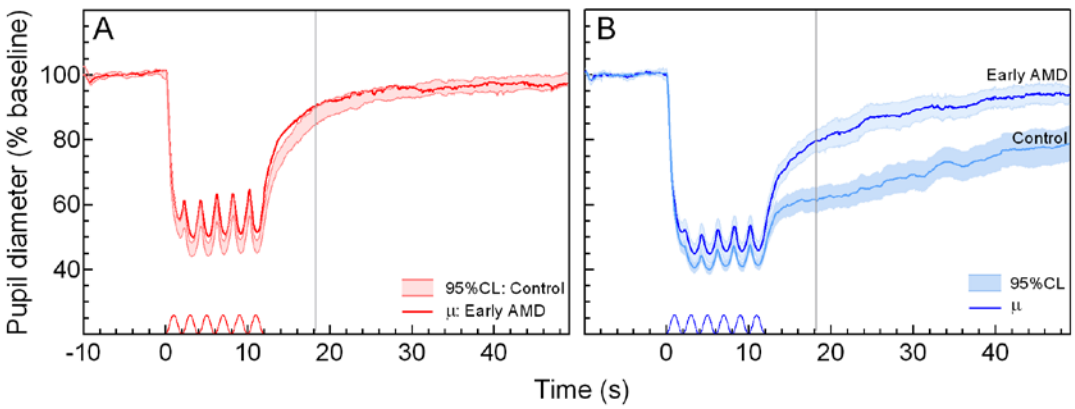


Figure 3

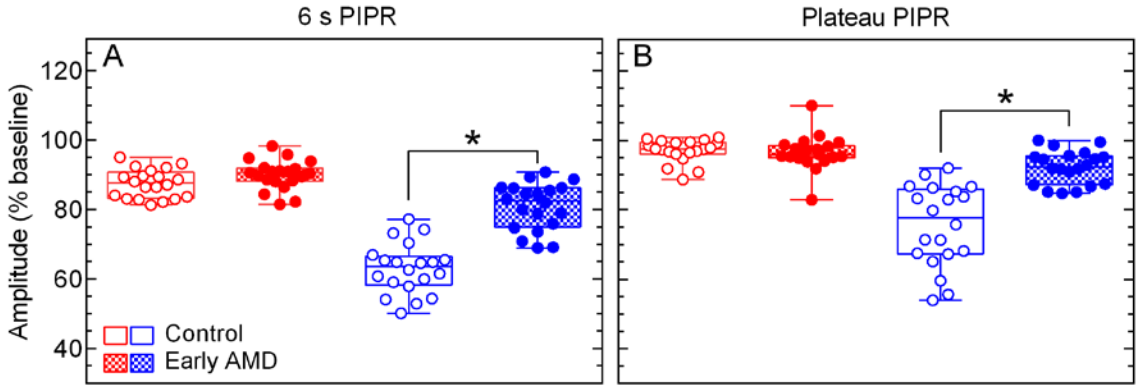


Figure 4

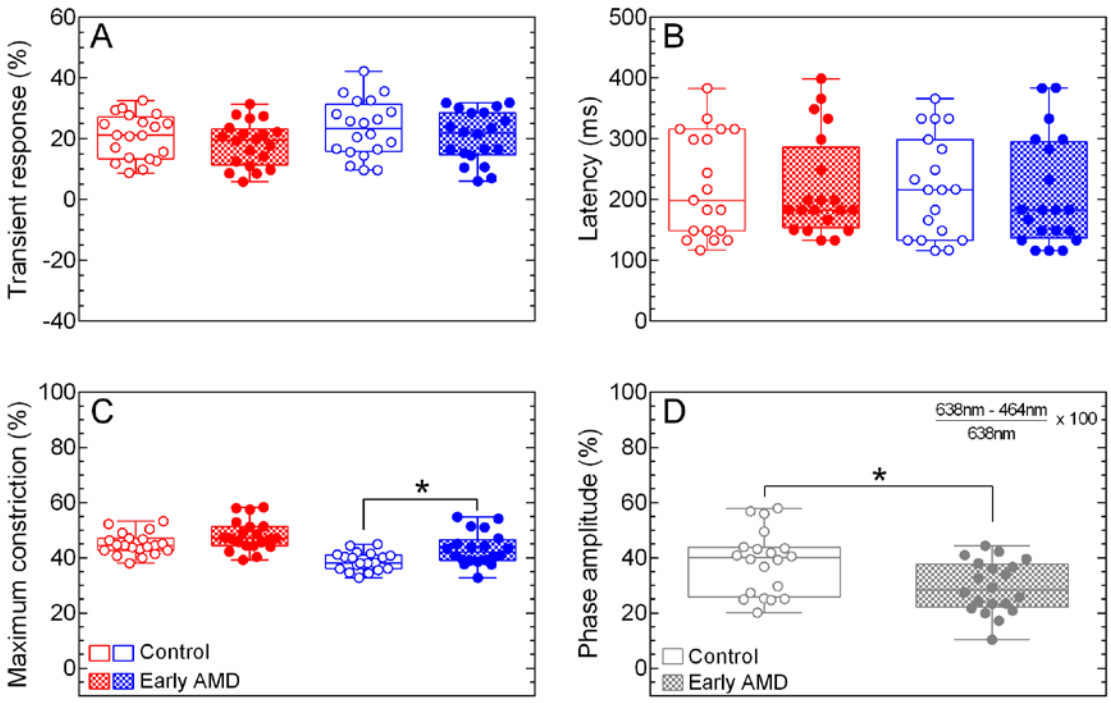
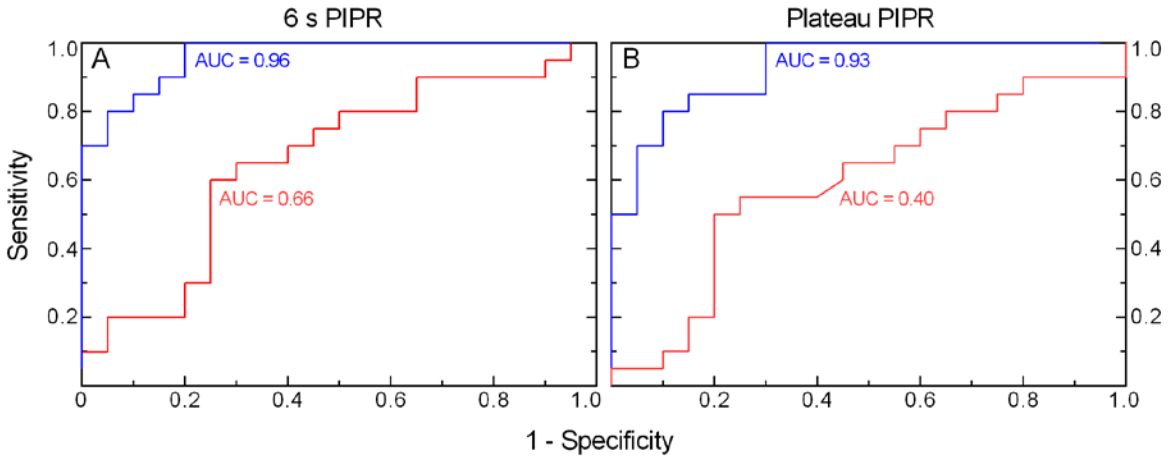


Figure 5


6 s PIPR
Plateau PIPR
A
B
AUC = 0.96
AUC = 0.66
AUC = 0.93
AUC = 0.40
Sensitivity
1 - Specificity
0
0.2
0.4
0.6
0.8
1.0